Lethbridge iGEM 2014 Standard Protocols:

Restriction Digest

Reagent	Volume
MilliQ	6 ul
10x Cutsmart Buffer	2 ul
DNA	10 ul
Enzyme 1	1 ul
Enzyme 2	1 ul
Total Volume	20 ul

Digest is put in 37C incubator for 3hrs – overnight. Restriction enzymes heat killed at 80C for 20 minutes

PCR

Standard PCR program:

1 – 98C for 5 minutes – Initial Denature

2 – 98C for 30 seconds – Denature

3 – Annealing temp* for 30 seconds – Annealing

4 – 72C for 1min/Kb (PFU) or 20s/Kb (Phusion) - Extension

5 – 72C for 5 minutes – Final extension

PFU

*Annealing temp for Pfu PCRs calculated by subtracting 5C from the Tm of the binding region of the primer. 5C is subtracted from the lowest of the pair of Tms

Reagent	Volume
MilliQ	18.9 ul
10x Pfu Buffer	3 ul
10mM dNTPs	0.6 ul
10uM F Primer	3 ul
10uM R Primer	3 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	30 ul

Phusion

*Annealing temperatures for Phusion PCRs are calculated using the NEB Tm Calculator (http://tmcalculator.neb.com/#!/)

Reagent	Volume
MilliQ	19.9 ul
5x HF Buffer	6 ul
10mM dNTPs	0.6 ul
10uM F Primer	1 ul
10uM R Primer	1 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	30 ul

Gel Extraction

Appropriately sized bands sliced from gel and DNA extracted according to Biobasic EZ-10 column protocol.

Ligation

Amount of Insert DNA to put in ligation calculated using this formula. All ligations were set up using a 4:1 insert to vector molar ratio with a maximum total of 100ng of DNA in a 10 ul reaction

$$ng\ insert\ DNA = \left(\frac{ng\ vector\ DNA * insert\ size\ (kb)}{vector\ size\ (kb)}\right) * \frac{4}{1}$$

Volumes of insert and vector DNA to add found using the quantities calculated using the above formula and measuring insert and vector concentration by nanodrop.

Reagent	Volume
MilliQ	To 10 ul total
NEB 10x Ligase buffer	1 ul
Insert DNA	X ul
Vector DNA	Y ul
T4 Ligase	0.5 ul
Total Volume	10 ul

Transformation

- 1.2 ul of the ligation mix added to 20ul of High efficiency NEBa competent cells
- Cells left on ice for 30 minutes
- Cells put in 42C water bath for 45 seconds
- Cells put back on ice for 5 minutes
- 400ul of SOC media added to cells
- Cells put in 37C shaking incubator for 1 hour
- 200 ul of cells plated on agar plates with appropriate antibiotic

Liquid Cultures

- 5ml LB culture tube spiked with 5ul of 1000x antibiotic stock
- Colonies or glycerol stocks touched with the end of a pipette tip
- Pipette tip swirled in culture tube. End of tube is flamed and capped
- Tube put in 37C shaking incubator for maximum 16 hours

Miniprep

Done according to instruction for Biobasic EZ-10 column instructions

Agarose Gel

For DNA fragments from 250bp to 8kb: samples ran on 1% agarose gel made with 1X TAE. Running buffer is 1X TAE. Ran for 30 minutes at 135v

Round the Horn Mutagenesis

This protocol is based off of the "Round the Horn Site-directed Mutagenesis" protocol by Sean Moore (http://openwetware.org/wiki/%27Round-the-horn_site-directed_mutagenesis)

Primer phosporylation

Reagent	Volume
MilliQ	32 ul
10x PNK buffer A	5 ul
25mM MgSO4	2 ul
100uM Primer	5 ul
20mM ATP	5 ul
PNK	1 ul
Total Volume	50 ul

Reaction is put in 37C incubator for 1 hour and heat killed at 95C for 5 minutes.

PCR reaction

Reagent	Volume
MilliQ	39 ul
10x Pfu Buffer	5 ul
10mM dNTPs	1 ul
10uM F Primer (phosphorylated)	1.5 ul
10uM R Primer (phosphorylated)	1.5 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	50 ul

The standard PCR program described above is used.

DpnI digest

1ul of NEB DpnI enzyme added to PCR product. Incubated at 37C for 3 hours and heat killed at 80C for 20 minutes

Recircularization of Digested PCR product

Reagent	Volume
MilliQ	2.3 ul
NEB 10x Ligase buffer	0.5 ul
DpnI digest PCR product	2 ul
T4 Ligase	0.2 ul
Total Volume	5 ul

Exosome Prep

Generating exosome free media

Complete media is pre-spun to remove vesicles present in Fetal Bovine Serum. 12 ml of media is put into polycarbonate vessels and spun at 125,000g for 70 minutes.

Exosomes Isolation by Centrifugation

Media from cells collected, spun at 300g for 10 minutes to remove non-adherent cells, and filtered using a 200nm filter.

Filtered media spun at 125,000g for 70 minutes. Supernatant is removed and the pellet is resuspended in 1X PBS or 2% PFA depending on intended use.

Isolated exosomes stored at 4C.